# Production and Maintenance of Conidia of *Gremmeniella abietina*

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**ABSTRACT**

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*Gremmeniella abietina* produced abundant conidia when grown on V-8 juice agar amended with a variety of nutrients and vitamins, especially when cultures were incubated in the light at 10–20 C. Germinability of conidia declined as cultures aged beyond 4 wk or as conidia were stored under water.

Scleroderris canker, caused by *Gremmeniella abietina* (Lagerb.) Morelet, was recognized as a killer of young conifers in North America in the early 1960s (4). Sanitation and rigorous fungicide applications, especially in nurseries, helped keep losses to a minimum (7). When a new strain of the pathogen was discovered in New York in 1973 (5), it caused considerable alarm. The new strain, subsequently labeled the European strain (ERS) (6), was of particular concern to forest managers because it killed trees of all ages and sizes. Control measures that were successful in nurseries were impractical in stands of older, larger trees.

For field inoculations reported previously (2), we were obliged to develop standard procedures for inoculum production and to ensure that the inoculum was viable when finally applied to seedlings. This paper reports experiments to determine conditions for consistent production and maintenance of conidia of *G. abietina*.

## MATERIALS AND METHODS

For most experiments, an isolate of *G. abietina* (GA 39) from a red pine (*Pinus resinosa* Ait.) growing near Harrisville, NY, and identified by serological tests as ERS was used. Other ERS isolates were also from northern New York. North American strain (NAS) isolates from jack pine (*P. banksiana* Lamb.) in Wisconsin were supplied by D. D. Skilling, U.S. Forest Service. D. R. Bergdahl supplied cultures of an intermediate strain (VIS) (8) from Vermont.

Isolates were grown on a medium similar to that used by Bruck et al (1) for culture of *Phytophthora infestans* (Mont.) de Bary, except 250 ml of V-8 juice was substituted for lima bean extract, and 20 g of Bacto agar was used instead of 15 g to make a *Gremmeniella* sporulation agar (GSA) (Table 1). For first-generation cultures, 300 ppm of streptomycin sulfate was added to GSA to eliminate contaminating bacteria (mostly *Pseudomonas* spp.) that otherwise inhibited conidial germination in subsequent tests (3).

Sporulation of *G. abietina* was assayed by flooding cultures with sterile distilled water (SDW) (10 ml per 9-cm-diameter petri dish), soaking them for 2 min, gently swirling the dishes for 30 sec, and determining the concentration of conidia that accumulated in the wash water. If the wash water was visibly turbid, transmission of light ($\lambda$ = 620 nm) through it was measured with a colorimeter and compared with a predetermined standard curve for suspensions of conidia of *G. abietina*. If the wash water was clear, concentrations of conidia were determined microscopically with a hemacytometer.

Germinability of conidia was determined by spreading 0.1-ml suspensions of conidia on 2% (w/v) Difco-Bacto agar (WA) in 9-cm-diameter petri dishes and incubating the dishes at 18 C in continuous light (1,500 lux). After 48 hr, plates were flooded with 0.002% aqueous cotton blue and examined microscopically. If a conidium had a germ tube at least as long as its widest portion, it was considered germinated.

**Sporulation of eight isolates on GSA.** Sporulation of each of the eight isolates was tested on GSA and on the following modifications thereof: GSA without vitamin stock, GSA without trace element stock, GSA without trace element or vitamin stock, and GSA without V-8 juice. Conidia from 3-wk-old cultures were spread on appropriate media and incubated in continuous light (2,000 lux) at 18 C for 3 wk. Sporulation was assayed as described earlier. Each treatment was replicated five times and the experiment was repeated twice.

**Effects of light and temperature on sporulation.** Conidia from 3-wk-old cultures of GA 39 were spread evenly onto GSA and incubated at 18 C. A fluorescent lamp attached to the ceiling of the incubator operated continuously, and cultures were exposed to light intensities ranging from 20 to 2,000 lux, depending on distance from the lamp. Some dishes were wrapped in aluminum foil to completely exclude light. Sporulation was assessed after 3 wk. Each treatment was replicated six times and the experiment was repeated once.

To determine the effect of photoperiod on sporulation, conidia of GA 39 were spread on GSA and incubated at 18 C under one of the following light regimes at 2,000 lux: continuous light, 8 hr of light/16 hr of darkness, 16 hr of light/8 hr of darkness, or continuous darkness. After 3 wk, sporulation was assessed. Each treatment was replicated three times and the experiment was repeated twice.

To determine the effect of temperature on sporulation, conidia of GA 39 were

**Table 1.** Modified *Gremmeniella* sporulation agar (GSA)

| Components | Amount |
|---|---|
| Major ingredients[a] | |
| Sorbitol | 5.0 g |
| Mannitol | 5.0 g |
| Dextrose | 5.0 g |
| $KNO_3$ | 3.0 g |
| $K_2HPO_4$ | 1.0 g |
| $KH_2PO_4$ | 1.0 g |
| $MgSO_4$ | 0.5 g |
| $CaCl_2$ | 0.1 g |
| Vitamin stock[b] | 2.0 ml |
| Biotin | 0.2 mg |
| Folic acid | 0.2 mg |
| i-Inositol | 12.0 mg |
| Nicotinic acid | 60.0 mg |
| Pyridoxine-HCl | 18.0 mg |
| Riboflavin | 15.0 mg |
| Thiamine-HCl | 38.0 mg |
| Coconut milk | 50.0 ml |
| Distilled water to make 300 ml | |
| Trace elements[b] | 2.0 ml |
| $FeC_6H_5O_7 \cdot 3\ H_2O$ | 215.0 mg |
| $ZnSO_4 \cdot 7\ H_2O$ | 150.0 mg |
| $CuSO_4 \cdot 5\ H_2O$ | 30.0 mg |
| $MnSO_4 \cdot H_2O$ | 15.0 mg |
| $H_3BO_3$ | 10.0 mg |
| $MoO_3$ | 7.0 mg |
| Distilled water to make 400 ml | |
| Yeast extract | 2.0 g |
| V-8 juice | 250.0 ml |
| Bacto agar | 20.0 g |
| Distilled water to make 1 L | |

[a] Autoclave for 30 min before dispensing into sterile containers.
[b] Keep refrigerated.

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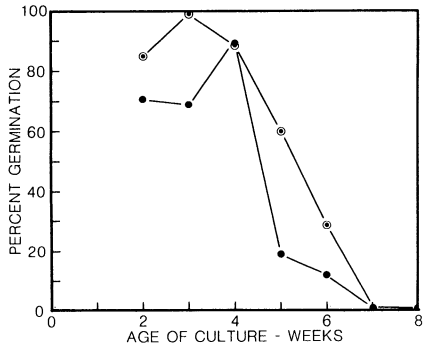


**Fig. 1.** Relationship of culture age to germinability of conidia of *Gremmeniella abietina* (GA 39). Results of two experiments are shown. Each point represents a mean of six replicates.

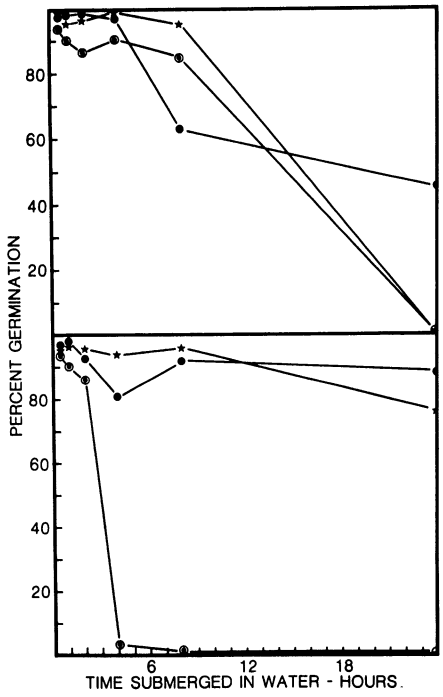


**Fig. 2.** Effect of immersion in sterile distilled water on germinability of conidia of *Gremmeniella abietina*. Results of two experiments are shown. Each point represents a mean of nine replicates. Temperatures: • = 10 C, ★ = 20 C, and ⊙ = 30 C.

spread on GSA and incubated for 3 wk in continuous light (2,000 lux) at one of four constant temperatures: 10, 15, 17, or 22 ± 1 C. Sporulation was then assayed. Each treatment was replicated five times and the experiment was repeated once.

**Viability of conidia related to culture age.** Conidia of GA 39 were spread on dishes containing GSA; the dishes were wrapped with Parafilm to retard drying and incubated in continuous light (2,000 lux) at 18 C. For 2–8 wk at weekly intervals, conidia were sampled and tested for germinability. For each sample, an 8-mm-diameter plug containing pycnidia with apparently fresh cirri was cut from each of six cultures, and each plug was placed in a test tube containing 1 ml of SDW. The tubes were shaken vigorously for 5 sec, and 0.1-ml samples of conidial suspensions therein were spread evenly onto WA to be assayed for germinability. The experiment was repeated twice.

**Survival of conidia stored in SDW.** Conidia from five 3-wk-old cultures of GA 39 were washed into a flask with SDW, and the resulting suspension was adjusted to $10^5$ conidia per milliliter. Then, 10-ml samples were removed to three lots of 21 test tubes and each lot was incubated in the dark at 10, 20, or 30 C. Three test tubes were removed from each lot at 0.5, 1, 2, 4, 8, 24, and 48 hr, and three 0.1-ml samples from each tube were spread onto WA plates to be assayed for germinability. The experiment was repeated twice.

## RESULTS

We noted that conidia of *G. abietina* did not germinate if submerged in water. Thus, care was taken to keep condensation on lids of WA dishes used in germination tests from falling onto the agar itself.

**Sporulation of eight isolates on GSA.** Concentrations of conidia in suspensions from various isolates growing on GSA did not differ significantly ($P = 0.05$), but there was considerable variation among dishes. Concentrations of conidia averaged $3.0 \times 10^7$ per milliliter and ranged from $9.0 \times 10^5$ to $6.3 \times 10^7$. Cultures of NAS and ERS isolates were relatively uniform in appearance, with many pycnidia and little aerial mycelium. However, cultures of VIS isolates were characterized by few large pycnidia and abundant fluffy, aerial mycelium.

Sporulation of various isolates on GSA prepared without vitamin stock and/or without trace elements did not differ significantly ($P = 0.05$) from that on standard GSA. However, GSA without V-8 juice supported little mycelium growth and no sporulation.

**Effects of light and temperature on sporulation.** Sporulation by isolate GA 39 in constant darkness was rare, but once an undefined minimum light requirement was met, variations in light intensity did not significantly affect spore production. Continuous light was not a requisite for sporulation. The shortest light period, 8 hr of light/16 hr of darkness, yielded an average of $5.2 \times 10^7$ conidia per milliliter and yield in continuous light averaged $8.4 \times 10^7$.

The concentration of conidia produced in culture at 10, 15, 17, or 22 C (pooled data of two replicates) ranged from $8.1 \times 10^5$ to $1.5 \times 10^7$ per milliliter (mean = $1.5 \times 10^7$).

**Viability of conidia related to culture age.** Sporulation was first observed in 10-day-old cultures, and most conidia from cultures up to 4 wk old germinated. However, conidia from cultures 5–8 wk old had a marked reduction in percentage of germination (Fig. 1) despite the relatively "healthy" appearance of the cultures. Germinability averaged 83% in weeks 2, 3, and 4 but dropped to an average of 39% in week 5 and was zero by week 8 (pooled data, two tests). Microscopically, conidia from some but not all older cultures differed from younger ones in that they had wrinkled rather than smooth cell walls and did not stain with cotton blue. Such conidia never were observed to germinate.

**Viability of conidia related to time immersed in water.** Typical relationships between germination and storage interval in SDW are illustrated in Figure 2. Survival of conidia in SDW was unpredictable after about 2 hr, and we observed zero germination after as few as 4 hr at 30 C and 24 hr at 20 C.

## DISCUSSION

GSA is an excellent medium for producing numerous conidia of ERS and NAS isolates relatively quickly, provided cultures are illuminated at least 8 hr daily. Our experience is that continuous fluorescent light (2,000 lux) and a temperature of 18 ± 1 C are best. When comparing sporulation in several dishes, it is important to ensure that all dishes receive similar light intensities. Do not pile plates one on top of another or indiscriminantly place them on different shelves.

Even though preparation of desired concentrations of conidial suspensions is most conveniently done in the laboratory, unpredictable survival of conidia in SDW for more than 4 hr precludes prior mixing of conidial suspensions for field inoculations. Thus, all preparation should be done at the site where inoculum is to be applied.


### ACKNOWLEDGMENT

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